

RECEIVED

AUG 2 8 2003 Vol. 55, No. 1

TECH CENTER 1600/2900

Site-Directed Mutagenesis of the Simian Virus 40 Large T-Antigen Gene: Replication-Defective Amino Acid Substitution Mutants That Retain the Ability to Induce Morphological Transformation

KEITH W. C. PEDEN¹* AND JAMES M. PIPAS²

Howard Hughes Medical Institute Laboratory, Department of Molecular Biology and Genetics, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205,¹ and Department of Biological Sciences, University of Pittsburgh, Pittsburgh, Pennsylvania 15260²

Received 25 October 1984/Accepted 5 March 1985

We used a heteroduplex deletion loop mutagenesis procedure for directing sodium bisulfite-induced mutations to specific sites on viral or plasmid DNA to generate a series of SV40 large T-antigen point mutants. The mutations were directed to a region of the T-antigen gene, 0.5 map units, that is thought to be important for interaction of the protein with the viral origin of DNA replication. Of the 16 mutants reported here, 10 had lost the ability to replicate their DNA, and 3 others showed a reduced level of replication compared to wild type. All of the mutants tested were capable of transforming rat cells in culture by the dense focus assay. We conclude that the sequences of the early region around 0.5 map units are critical for the replication of viral DNA but not for the transformation function of T antigen.

The large tumor antigen (T antigen) of simian virus 40 (SV40) is a regulatory protein that is expressed early after infection and functions to initiate viral DNA replication (71), activate late region transcription (1, 12, 25; 26, 45, 55), and autoregulate early region transcription (1, 26, 53, 73). In addition, T antigen is capable of inducing the morphological transformation of a number of cell types in culture (3, 6, 19, 27, 28, 38, 44, 59, 68, 70, 72). During infection of both permissive and nonpermissive cells, T antigen induces a number of changes in cellular metabolism. For example, T antigen stimulates cellular DNA synthesis (7, 15, 21, 22, 57, 65, 75) and activates the transcription of certain cellular genes (64-66).

Three biochemical activities of T antigen have been described: a DNA-binding activity (41, 42, 52, 58, 74), an ATPase activity (8, 16, 76), and the ability to bind a host protein of 53,000 daltons, p53 (30, 33). The binding of T antigen to specific sequences at the origin of viral replication is thought to be required for the autoregulation of early region transcription, the stimulation of late region transcription, and the initiation of viral DNA replication. Furthermore, systems that replicate SV40 DNA in vitro are dependent upon T antigen (2, 32). The activities that function to induce morphological transformation have not been identified.

One approach to understanding the molecular mechanism of T antigen action is to correlate the biochemical activities of this molecule with its multiple biological effects. To this end, we have attempted to separate the activities of T antigen by mutating the T-antigen gene at specific sites. Experiments with deletion mutants that synthesize fragments of the protein have demonstrated that some activities can be dissociated (9, 15, 49, 65). These studies also indicated that certain activities seemed to reside in discrete functional domains of the protein. For example, the ability to

stimulate cellular DNA synthesis requires residues between amino acids 82 and 272 and is unaffected by mutations outside this region (65). Similarly, the function that activates the transcription of silent rRNA genes resides in the central portion of the protein (65). On the other hand, the abilities to initiate viral DNA replication and to induce morphological transformation of cells in culture were sensitive to mutations in several regions of the molecule and were not dissociated from each other (49). This indicates that either the same biochemical activities of T antigen that induce transformation are required for viral DNA replication or the mutational lesions alter the protein structure such that independent activities are lost coordinately.

Results from Shortle et al. (62) and Margolske and Nathans (37) have implicated the region of SV40 T antigen around 0.5 map units (m.u.) as being involved in viral DNA replication. Mutations at the origin of DNA replication leading to a reduced ability to replicate could be suppressed by mutations in T antigen, and two second-site revertants have been found to have lesions that map to 0.5 m.u. on the SV40 genome, changing amino acids 157 and 166, respectively. Additional evidence that this region of the protein is involved with the DNA replication activity has come from the studies of Stringer (69), Gluzman and Ahrens (18), and Manos and Gluzman (35, 36). Viral genomes isolated from SV40-transformed monkey cells (18, 35, 36) or rat cells (69) have been found to carry mutations in the T antigen gene that eliminate the ability of the protein to replicate its DNA while maintaining the transformation activity. Therefore, to determine whether in vitro-constructed mutations in this region would affect the DNA replication activity, and also to map the extent of this region, it was decided to target 0.5 m.u. of SV40 for site-directed mutagenesis. With a series of mutants it should be possible to characterize which biochemical activities are involved with DNA replication and transformation. A similar approach has been taken recently by Kalderon and Smith (24). We report here the isolation and characterization of in vitro-constructed amino acid substitu-

* Corresponding author.

tion mutants that eliminate the DNA replication function of T antigen while leaving the transformation activity relatively unaffected.

MATERIALS AND METHODS

Cells, viruses, DNAs, and bacterial strains. The small plaque strain of SV40 (776) was grown in BSC40 cells, as described previously (4, 5). REF52 and Rat-1 (79) cells were obtained from W. Topp, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., and grown in Earle minimum essential medium supplemented with 10% fetal bovine serum (HyClone; Sterile Systems, Inc., Logan, Utah). SV40 deletion mutants are described by Pipas et al. (49) and in references within their study. The plasmid pKP38 is pBR322 from which the *Ava*I and *Sal*I sites have been mutated by repairing the four unpaired nucleotides, followed by ligation, as described by Peden and Nathans (47). MM294 and BD1528 have been described previously (43, 47).

Preparation of plasmid DNA. Small-scale preparations of plasmid DNA were made by the method of Holmes and Quigley (23). Large-scale preparations were carried out as described previously (46, 47).

Construction of nucleotide substitution mutations. The procedure used followed closely that described previously (47). DNA from the plasmid pSVB40, a recombinant of wild-type SV40 and pKP38 joined through their *Bam*HI sites, was digested with *Kpn*I, and DNA from pSVdl1137 and pSVdl1138 was digested with *Cla*I. *Kpn*I-digested pSVB40 (2 µg) was mixed with *Cla*I-digested pSVdl1137 (2 µg) and, separately, with *Cla*I-digested pSVdl1138 (2 µg) in 1,620 µl of H₂O, denatured by addition of 1 M NaOH (180 µl; final concentration, 0.1 M) and incubation at 37°C for 10 min, and neutralized by addition of 360 µl of neutralizing solution (1 M Tris hydrochloride [pH 7.2], 1 M HCl; 2:1; see reference 29). Renaturation was allowed to proceed at 60°C for 2 h. The extent of heteroduplex formation was determined by agarose gel electrophoresis, since these molecules migrate with form II species, and varied between 20 and 40% of the linear, homoduplex molecules. This mixture was concentrated by ethanol precipitation and dissolved in 200 µl of 10 mM Tris hydrochloride (pH 8)–1 mM EDTA.

Mutagenesis with sodium bisulfite was carried out as described previously (17, 47, 60). Between 50 ng and 1 µg of the heteroduplex mixture was incubated with 2.5 M sodium bisulfite–1 mM hydroquinone at 37°C under paraffin oil in the dark for 1 h, followed by dialysis by the method of Shortle and Nathans (60). This mutagenized DNA was used directly to transform BD1528 to ampicillin resistance (31), and plasmid DNA was prepared from the pooled transformants (23). Full-length molecules were enriched by digestion of the deletion mutant DNA with *Ava*I and *Sal*I, and DNA was prepared from individual MM294 transformants. Those full-length molecules (ascertained by restriction enzyme mapping; *Hind*III plus *Taq*I for the pSVdl1137 series, *Hin*fI for the pSVdl1138 series) were subjected to sequence analysis. Fifty to 80% of those molecules sequenced had nucleotide changes.

DNA sequence analysis. Mutants were subjected to sequence analysis by the method of Maxam and Gilbert (39) after labeling the 3' ends of restriction fragments with *Micrococcus luteus* DNA polymerase I and the appropriately labeled [α -³²P]dNTP and unlabeled dNTP. Cleavage products were resolved on 8% polyacrylamide gels (56). Mutants in the dl1137 region were labeled with [α -

³²P]dGTP at the *Dde*I site at nucleotide 4499 (78), and the fragments were separated by electrophoresis on 6% polyacrylamide gels and recovered by the method of Maxam and Gilbert (39). Mutants in the dl1138 region were labeled with [α -³²P]dCTP at the *Hin*fI site at nucleotide 4376, and the fragments were separated by electrophoresis on 2.5% agarose gels and recovered on NA45 membranes by a protocol of Lizardi supplied by Schleicher & Schuell, Inc., Keene, N.H.

Indirect immunofluorescence in monkey cells. BSC40 cell monolayers in 5-cm dishes were transfected with 1 µg of plasmid DNA from the mutants by the DEAE-dextran method (40). The next day, the cells were trypsinized, plated in chamber slides, allowed to attach, and fixed in ethanol. T antigen was detected with hamster SV40 anti-T serum (50).

Viability and complementation tests. Viability was determined at 32, 37, and 40°C by transfecting BSC40 cell monolayers with 1 ng of excised and ligated DNA per 5-cm dish by the DEAE-dextran method (40). Plaques were scored at 32°C between 3 and 4 weeks and at 37 and 40°C between 12 days and 3 weeks.

Complementation tests were done by coinfecting BSC40 cell monolayers with mixtures of 10 ng each of excised and ligated mutant DNA and dl1007 DNA per 5-cm dish. Plaques were scored after 12 to 14 days at 37°C.

Marker rescue. Marker rescue was performed by using modifications of published procedures (18, 29). SV40 fragments used for the marker rescue were either cloned into pBR322 or purified from agarose gels by electroelution and DEAE-cellulose chromatography (63). Wild-type viral *Hind*III fragments were inserted into the *Hind*III site of pBR322, *Hae*III fragments were inserted into the *Bal*I site, and *Alu*I fragments were inserted into the *Pvu*II site of pBR322. *Bam*HI-digested mutant DNA (30 to 50 ng), *Hae*III-digested plasmid containing the SV40 *Hae*III C fragment (120 ng), and either the appropriately digested plasmid containing the fragment to be tested (120 ng) or the gel-purified fragment (10 to 20 ng) were mixed and then denatured by addition of 0.1 volume of 1 M NaOH and incubation at 37°C for 10 min. After neutralization by addition of 0.2 volume of neutralizing solution (see above), heteroduplex formation was allowed to occur (in a final volume of 260 µl) by incubation at 68°C for 30 min. DEAE-dextran (260 µl) was added to 250 µg/ml, and the mixture was used to transfect two 5-cm dishes of BSC40 monolayers. After 1 to 2 h at 37°C, minimum essential medium plus 10% fetal bovine serum was added, and the cells were incubated at 37°C overnight. The following day, the medium was replaced with agar for plaque determination as described above.

DNA replication in monkey cells. Viral DNA replication of the mutants was measured in BSC40 cells as described previously (48, 49). After digestion with *Dpn*I plus *Bcl*I, DNA was fractionated by electrophoresis on 1% agarose gels and transferred to nitrocellulose (67), and viral sequences were detected by hybridization with ³²P-labeled SV40 DNA (labeled by nick translation [34, 54]). Hybridization and washing of filters were done as described previously (46).

Transformation of rat cells. Rat embryo fibroblasts (REF52) and Rat-1 cells (79) were seeded into 5-cm dishes at approximately 5×10^4 cells per dish the day before transfection. Mutant DNA (1 µg per dish) was introduced, using the calcium phosphate procedure (20) and glycerol shock (14). Dishes were fed every 3 days with minimal essential medium plus 10% fetal bovine serum, and foci were scored at 3 weeks after transfection.

RESULTS

Mutant construction. Base substitution mutations were generated in the T antigen gene at 0.5 m.u. by the heteroduplex deletion loop mutagenesis method described previously (47). In this method, a heteroduplex is prepared between a deletion mutant and a wild-type molecule, and the cytosine residues in the resulting single-stranded loop at the position of the deletion become targets for deamination by sodium bisulfite. Two deletion mutants, dl1137 and dl1138 (49), were used as templates for mutant construction. The procedure is described above and outlined in Fig. 1.

Sequence determination of mutants. Figures 2 and 3 show the nucleotide sequence changes together with the predicted amino acid substitutions of mutants constructed with dl1137 and dl1138 as templates, respectively. Both cytosine-to-thymine and guanine-to-adenine transitions were found, as expected, since both strands are able to participate in heteroduplex formation. Of the 11 cytosine-guanine base pairs (bp) susceptible to sodium bisulfite in the exposed 31 nucleotides of dl1137, 8 were changed. More importantly, of the six amino acids that could be changed by this method, four were mutated. In the case of the dl1138 series, 11

TABLE 1. Infectivity of SV40 point mutants

Mutant	Infectivity (PFU per dish) at the following temp (°C)			
	32	37	40	37 ^a
pSVB3	101	109	98	ND ^b
5013	91	103	97	112
5014	0	0	0	86
5015	50 ^c	64 ^c	61 ^c	76
5016	53 ^c	42 ^c	35 ^c	42
5017	0	0	0	53
5018	0	0	0	50
5020	0	0	0	63
5021	60	76	81	70
5022	0	0	0	42
5023	0	0	0	55
5024	83 ^d	107 ^d	122 ^c	85
5025	0	0	0	47
5026	0	0	0	76
5027	0	0	0	38
5028	0	0	0	54
5032	77	81	82	ND

^a dl1007 added.

^b ND, Not done.

^c Small plaque morphology.

^d Small plaque morphology, appearing several days late.

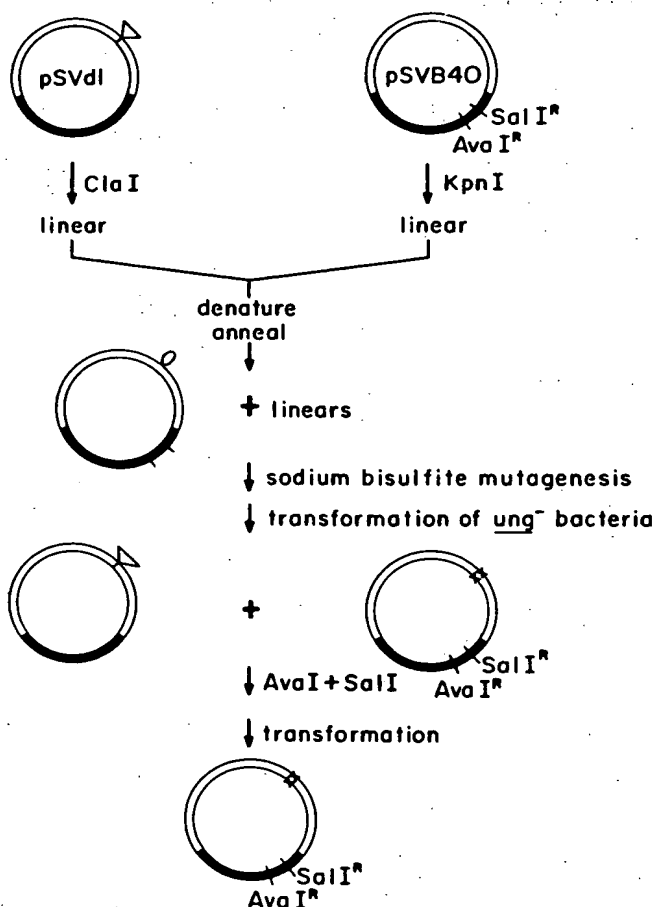


FIG. 1. Summary of mutagenesis procedure. The plasmid pSVdl is a recombinant between an SV40 deletion mutant and pBR322, and pSVB40 is a recombinant between wild-type SV40 and a pBR322 derivative from which the *AvaI* and *SalI* sites were removed by mutation. The details are given in the text.

cytosine-to-guanine bp were targets and 10 were mutated, resulting in the changing of five of the six susceptible amino acids. Those mutations that did not change the amino acid sequence are not included here, since several of this class of mutant had no detectable phenotypic alterations.

Cellular localization of T antigen by indirect immunofluorescence. The ability of the mutants to produce a stable T antigen was determined by indirect immunofluorescence with a hamster polyclonal SV40 anti-T serum after transfection of BSC40 monolayer cultures with plasmid DNAs. All mutants produced nuclear fluorescence with intensities approximately that of the wild-type SV40 plasmid, pSVB3. Mutants 5017, 5018, and 5028 produced weak cytoplasmic fluorescence as well.

Viability on BSC40 cells. The ability of each mutant to plaque on BSC40 cell monolayers was measured at 32, 37, and 40°C, and the ability to complement the late deletion mutant helper, dl1007, was determined at 37°C (Table 1). None of the mutants except 5024 showed any temperature-dependent growth. Three mutants were viable and produced plaques with morphology indistinguishable from that produced by wild-type SV40 (5013, 5021, 5032), and two mutants produced small plaques (5015, 5016). Mutant 5024 showed a slight cold-sensitive phenotype, producing very small plaques 4 to 5 days later than wild type at 32°C and 1 to 2 days later at 37°C.

All defective and partially defective mutants could be complemented by dl1007, demonstrating that they are defective only in early function. Complementation plaques with the nonviable point mutants were smaller than those obtained with an early deletion mutant that produces no T antigen, such as dl1136 (49). This could be due to interference with wild-type T antigen function by the mutant protein.

Marker rescue of mutants. To confirm that the phenotype of the mutants was a consequence of the nucleotide changes determined by DNA sequence analysis, marker rescue experiments were carried out. The locations of the fragments used and of the deletions in dl1137 and dl1138 are shown in

FIG. 2. Sequences of point mutants isolated by using dl1137 as template. The nucleotide sequence of SV40 DNA together with the corresponding amino acid sequence of T antigen from amino acid residues 121 to 133 is shown across the top. Nucleotides 4454 and 4422 are the limits of the deletion of dl1137. Mutant designations are in the left column, and the nucleotide changes for each mutant are shown. Amino acid substitutions are underlined.

FIG. 3. Sequences of point mutants isolated by using dl1138 as template. The format of the figure is as described for Fig. 2.

Fig. 4. Mutations in both the 1137 and 1138 regions were rescued (Table 2) by both the *HindIII* B fragment (nucleotides 5171 to 4002) and the *RsaI* B fragment (nucleotides 4876 to 4168) but not by *HaeIII*-C (nucleotides 2799 to 2259; the fragment used to cyclize the heteroduplex), *HaeIII*-E (nucleotides 5191 to 4862), or *HindIII*-D (nucleotides 4002 to 3476; data not shown), thus localizing the lesions to the *RsaI* B fragment. The *AluI* C fragment (nucleotides 4643 to 4314) rescued mutants 5014, 5017, and 5018, which were isolated by using dl1137 as template, and some of the mutants isolated in the dl1138 region (mutants 5024, 5026, and 5027). Since the end of the *AluI* C fragment lies within the deletion limits of 1138, it would be anticipated that not all the mutants would be rescued. In fact, mutants 5023 and 5025, which have mutations causing amino acid changes either outside or at the last nucleotide of the *AluI* C fragment, are not rescued. In the case of mutants 5020, 5022, 5026, and 5028, which have mutations leading to amino acid changes both within and outside *AluI*-C, only 5026 is rescued by *AluI*-C, indicating that the substitution Ala-169 to Val is inconsequential. This was confirmed by obtaining DNA from two such rescuants and determining their DNA sequence. Both had the original cytosine-to-thymine transition at nucleotide 4312 of mutant 5026; this viable mutant is number 5032 in Fig. 3.

HinfI-C (nucleotides 4376 to 3610) failed to rescue the lesions in the 1137 series mutants but rescued all those in the 1138 series, as predicted (Table 2). Thus, the mutations in the 1137 series map to 267 bp between nucleotides 4643 and 4376, and those in the 1138 regions to 208 bp between nucleotides 4376 and 4168, or, if rescued by *AluI*-C, to the 62 bp between nucleotides 4376 and 4314. Since over 100 nucleotides of each mutant were sequenced and no additional alterations were found, we feel confident that the nucleotide substitutions of each mutant presented in Fig. 2 and 3 are responsible for the observed phenotypes.

DNA replication in BSC40 cells. To test whether the mutants retained the ability to replicate their DNA, we used the *DpnI* assay described previously (48, 49). Examples of this assay are shown in Fig. 5, in which the replication efficiencies of wild-type SV40 and mutants 5024, 5018, 5015, and 5016 are compared. In 10 out of 16 cases, no DNA

TABLE 3. Summary of biological activities^a

Mutant	Amino acid substitutions	Viability	Replication (% of wild type)	Transformation (% of wild type)
5013	Arg-130 → Lys	+ ^b	100	14
5014	Pro-125 → Ser	—	0	110
5015	Pro-126 → Leu	+ ^c	20	58
5016	Pro-126 → Ser	+ ^c	15	37
5017	Pro-125 → Ser, Pro-126 → Ser	—	0	115
5018	Ser-123 → Phe	—	0	60
5020	Thr-164 → Ile, Ala-168 → Val	—	0	32
5021	Ala-169 → Thr	+ ^b	100	57
5022	Thr-164 → Ile, Ala-168 → Val, Ala-169 → Val	—	0	15
5023	Ala-168 → Thr	—	0	58
5024	Glu-166 → Lys	+ ^d	40	23
5025	Ala-168 → Val	—	0	19
5026	Thr-164 → Ile, Ala-169 → Val	—	0	60
5027	Thr-163 → Ile, Thr-164 → Ile	—	0	40
5028	Thr-163 → Ile, Thr-164 → Ile, Ala-168 → Val	—	0	10
5032	Ala-169 → Val	+ ^b	ND ^e	108

^a Viability was measured as the ability to form plaques on monolayers of BSC40 cells at 37°C; replication was measured in BSC40 cells at 37°C, and transformation was measured by dense focus assay on rat cell lines REF52 and Rat-1. The numbers of foci per dish on REF52 cells varied between 80 and 110 with the wild-type SV40-pBR322 recombinant pSVB3, but on Rat-1 cells the numbers were 20 to 30 foci per dish. Data are shown only for the REF52 cell line. Foci were present in assays on Rat-1 cells with all of the mutants listed except 5032, which was not assayed.

^b Wild-type plaque morphology.

^c Small plaque morphology.

^d Small plaque morphology, slightly cold sensitive.

^e ND, Not done.

replication was detectable. Those mutants that produced plaques (5013, 5015, 5016, 5021, 5024) all replicated their DNA as expected. Since mutant 5032 produced plaques of wild-type morphology, it would also be expected to be positive in this assay although it was not tested. Mutant 5024, which formed late-appearing small plaques at 37 and 32°C, replicated its DNA to approximately 40% that of wild type at both temperatures (Table 3).

Morphological transformation of nonpermissive rodent cells. The ability of the T-antigen mutants to transform nonpermissive cells in culture was tested on two rat cell lines, the REF52 line and the Rat-1 line, using the dense focus assay of Todaro and Green (77). All mutants, whether viable or not, were able to induce foci with various efficiencies on both cell lines (Table 3). Thus, the transformation function by this assay is relatively insensitive to mutations in the region of the T-antigen gene between nucleotides 4450 and 4429 and nucleotides 4330 and 4312.

DISCUSSION

We report the isolation and preliminary characterization of 16 new SV40 mutants with amino acid substitutions in the large T antigen. The mutations were targeted to a region near 0.5 m.u. on the viral genome, using the heteroduplex deletion loop mutagenesis procedure for directing sodium bisulfite-induced transitions. Nucleotide changes were determined by DNA sequence analysis, and in those cases in which a mutant exhibited an altered phenotype, marker rescue experiments were carried out to confirm the locations of the mutations responsible for the phenotype.

TABLE 2. Marker rescue results

Mutant	Plaques per dish produced by the following restriction fragment				
	<i>HaeIII</i> -C	<i>HindIII</i> -B	<i>RsaI</i> -B	<i>AluI</i> -C	<i>HinfI</i> -C
5013	>100, >100	ND ^a	ND	ND	ND
5014	0, 0	51, 43	29, 33	35, 26	0, 0
5015	>100, >100 ^b	29, 18	ND	ND	ND
5016	>100, >100 ^b	17, 33	ND	ND	ND
5017	0, 0	28, 37	11, 13	19, 22	0, 0
5018	0, 0	25, 31	14, 4	20, 25	0, 0
5020	0, 0	23, 35	17, 40	0, 0	14, 3
5022	0, 0	32, 17	49, 23	0, 0	18, 15
5023	0, 0	26, 29	9, 16	0, 0	6, 7
5024	>100, >100 ^b	38, 45 ^c	21, 27 ^c	27, 23 ^c	10, 3 ^c
5025	0, 0	21, 19	30, 28	0, 0	19, 23
5026	0, 0	42, 26	40, 42	10, 19	9, 8
5027	0, 0	61, 38	24, 40	29, 28	9, 9
5028	0, 0	24, 30	60, 35	0, 0	8, 8

^a ND, Not done.

^b Small plaque morphology.

^c Numbers of wild-type plaques; ~100 small plaques appeared 2 to 3 days late.

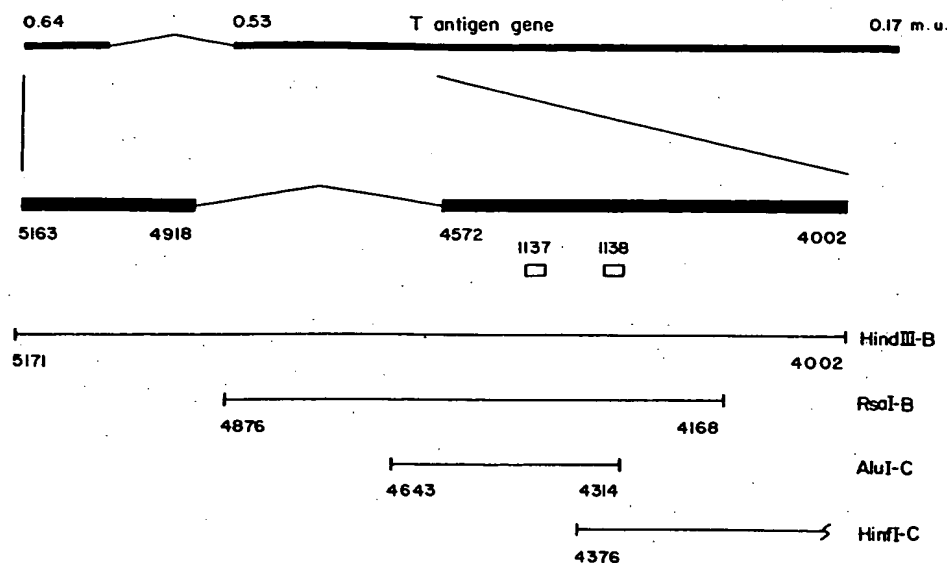


FIG. 4. Map positions of the fragments used for marker rescue. The T-antigen gene from m.u. 0.64 to 0.17 is shown at the top, and the region from nucleotide 5163 (0.64 m.u.) to nucleotide 4002 (0.42 m.u.) is expanded. The open boxes represent the locations of the deletions in dl1137 and dl1138, and the extent and nucleotide limits of the fragments found to marker rescue the mutations of the point mutants are shown.

Three of the mutants (5013, 5021, 5032) had normal plaque morphologies and, where tested, replicated their DNA to levels similar to that of wild-type SV40. These mutants were also capable of inducing dense foci on REF52 cells. We conclude that substitutions of Lys for Arg at position 130 and of either Val or Thr for Ala at position 169 do not alter the ability of T antigen to support productive viral infection or transformation.

Three mutants (5015, 5016, 5024) displayed a reduced ability to replicate their DNA as well as an altered plaque morphology. Mutants 5015 and 5016 change Pro-126 to Leu or to Ser, respectively. Both formed small plaques relative to wild type at the three temperatures tested. Therefore, the ability to undergo a productive infection is reduced but not abolished in these mutants.

The mutation in 5024 results in the substitution of Lys for Glu at position 166. This same residue is changed to Gln in a mutant selected as a pseudorevertant of an origin-defective mutant (37, 62). Since the revertant produces a T antigen capable of functioning with a number of mutant origins (61) that are defective with the wild-type T antigen (37), it indicates that this region of the protein is important for the interaction with the origin of replication. Mutant 5024 has a cold-sensitive phenotype, forming small plaques that appear late at 32°C. Viral DNA replication is reduced to about 40% of the level of wild-type SV40 at both 32 and 37°C. Since 5024 is capable of replicating its DNA at 32°C but forms very small plaques at this temperature, we suggest that the T antigen of 5024 is defective in an essential function for productive infection other than DNA replication, such as activation of late transcription, processing or translation of late mRNA, or encapsidation.

The remaining 10 mutants were defective for plaque formation at all three temperatures tested, and all failed to replicate their DNA to a detectable level. Two of these mutants, 5014 and 5017, contain a substitution of a Ser for Pro at position 125: mutant 5014 carries the single substitution while 5017 has an additional change of Pro-126 to Ser.

Thus, while replacement of Pro-126 with either Leu (mutant 5015) or Ser (mutant 5016) leads to a partially active protein, substitution of the adjacent Pro-125 with Ser results in a T antigen unable to replicate its DNA and unable to form plaques. Similarly, a substitution of Phe for Ser at position 123 (mutant 5018) and either Thr (mutant 5023) or Val (mutant 5025) for Ala-168 leads to the DNA replication-negative phenotype. Thr-164 may be critical for the replication function of T antigen, since mutant 5026, which carries the double substitution Thr-164 to Ile and Ala-169 to Val, is defective, while mutant 5032, which has the single substitution at position 169, is viable. Two mutants (5027, 5028) carry substitutions of Ile for Thr-163. However, the importance of this change cannot be assessed, since both also have the lethal replacement Thr-164 to Ile. In summary, we obtained amino acid substitutions at nine positions. Alterations at four of these residues, Ser-123, Pro-125, Thr-164, and Ala-168, destroyed the ability of T antigen to support the replication of viral DNA. Substitutions at another two positions, Pro-126 and Glu-166, reduced the level of viral DNA replication relative to wild type. Therefore, the replication function of large T antigen is very sensitive to mutations in this region of the protein.

In contrast, the ability of SV40 large T antigen to induce cellular transformation was not affected appreciably in any of the mutants so far tested. Of the 10 mutants that showed no detectable viral DNA replication in monkey cells, all were capable of transforming the REF52 rat cell line by the dense focus assay. We conclude that at least one of the activities of T antigen necessary for DNA replication in permissive cells is not obligatory to induce the transformed phenotype in nonpermissive cells and, therefore, the two activities are separable by mutation.

This conclusion is supported by the findings of others (18, 36, 39) that SV40 genomes isolated from transformed cells frequently possess point mutations between 0.45 and 0.5 m.u. of the T-antigen gene that result in the same phenotype. Replacements of Thr for Asn-153 (18), Gln for His-203 (36),

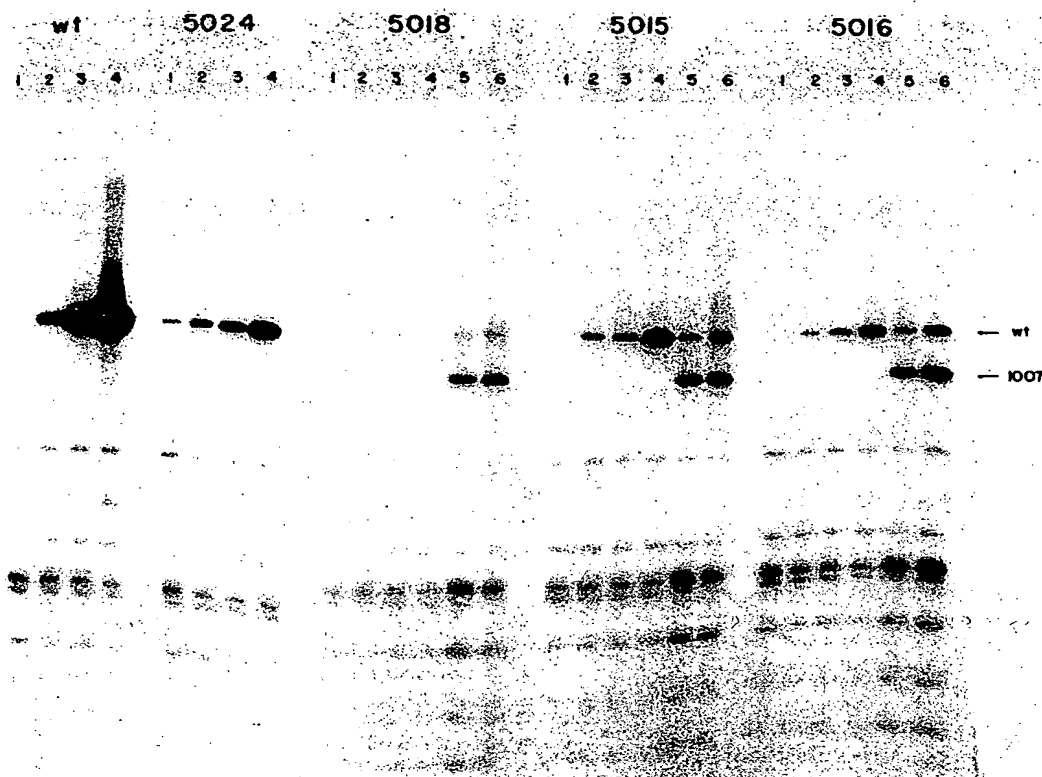


FIG. 5. Replication of SV40 point mutant DNA in monkey cells. Subconfluent BSC40 cell monolayers were transfected with point mutant DNA or point mutant DNA plus helper dl1007 DNA. Hirt DNA was prepared (48, 49) at different times after infection, purified, digested with *DpnI* and *BclI*, fractionated by electrophoresis on 1.2% agarose gels, transferred to nitrocellulose, and hybridized with ^{32}P -labeled SV40 DNA. DNA was prepared at the following times posttransfection: 24 h (lanes 1), 36 h (lanes 2), 48 h (lanes 3), 72 h (lanes 4) 36 h with dl1007 added (lanes 5), 48 h with dl1007 added (lanes 6).

Glu for Lys-214 (69), and Glu for Lys-224 (36) also conferred the replication-negative, transformation-competent phenotype. Furthermore, Pearson-White and Nathans (personal communication) constructed a mutant, 1153, containing an additional Ile residue inserted between amino acids 143 and 144. This mutant also possessed the same phenotype.

Recently, Kalderon and Smith (24) described an extensive series of mutants constructed *in vitro* by a method analogous to the one used here and reached similar conclusions. From the combined results, it can be concluded that the region of T antigen where mutations can lead to the replication negative, transformation-competent phenotype can be extended to include amino acids 106 to 224. It remains possible, of course, that mutations in other regions of the protein will also result in this phenotype. In fact, Manos and Gluzman (35) have shown recently that substitution of Arg for Lys-516 or of Ser for Pro-522 results in the same phenotype and, therefore, this part of T antigen must be involved, either directly or indirectly, in DNA replication.

While the molecular basis for the mutational separation of the transformation and replication functions of T antigen is not understood, the simplest possibility is that these mutations destroy a biochemical activity of T antigen required for viral DNA replication while leaving those activities that mediate transformation untouched. A detailed examination of the biochemical properties of the mutant proteins will be necessary to differentiate between the possible mechanisms. Of interest in this regard will be whether the mutant T

antigens bind specifically to the origin of SV40 DNA replication. Experiments with other replication-negative, transformation-competent mutant T antigens have shown that some are able to bind in a McKay assay (41) while others are not (36, 51), suggesting that the inability to bind to the origin of DNA replication cannot be the only mechanism that results in the replication-negative phenotype.

Mutants have been isolated that replicate their DNA but have a reduced potential to induce morphological transformation of nonpermissive cells. Cosman and Trevethia (11) described a mutant, *tsA1642*, that is temperature sensitive for lytic growth, replicates its DNA, but is unable to transform mouse embryo fibroblasts. Mutant 5002, which has two amino acid substitutions in the first exon of T antigen, is unable to transform REF52 cells but replicates its DNA to about 50% of wild-type levels (K. W. C. Peden, unpublished observations). These two mutants suggest that the replication activity may not be sufficient for the transformation of nonpermissive cells.

Finally, we point out that site-directed mutagenesis provides a powerful tool for introducing changes into regions of proteins thought to be of functional significance. Such regions may be identified by virtue of their primary structure or indicated by previous genetic analysis. Thus, specific hypotheses concerning structure-function relationships can be tested. Along these lines, we are currently directing mutations to other parts of T antigen such as the amino acids known to be phosphorylated (80), the regions conserved

among the various papovavirus T antigens (13), the residues to which the ATPase activity has been localized (8, 10), and the major hydrophobic region of the protein.

ACKNOWLEDGMENTS

We thank Daniel Nathans for continued interest and support and for comments on the manuscript. In addition, we would like to express gratitude to the participants of the Cedar Bay Conference for lively criticism of the work, to Laura Sanders and Fredda Slutzman for able technical assistance, to Jacque Migeon for sequencing some of the mutants, and to Doris Wiczulis and Lily Cowan for typing the manuscript.

This work was supported by Public Health Service grant 5 P01 CA16519 from the National Cancer Institute and grant MV-182 from the American Cancer Society.

LITERATURE CITED

- Alwine, J. C., S. I. Reed, and G. R. Stark. 1977. Characterization of the autoregulation of simian virus 40 gene A. *J. Virol.* 24:22-27.
- Ariga, H., and S. Sugano. 1983. Initiation of simian virus 40 DNA replication in vitro. *J. Virol.* 48:481-491.
- Brockman, W. W. 1978. Transformation of BALB/c-3T3 cells by *tsA* mutants of simian virus 40: temperature sensitivity of the transformed phenotype and retransformation by wild-type virus. *J. Virol.* 25:860-870.
- Brockman, W. W., T. N. H. Lee, and D. Nathans. 1973. The evolution of new species of viral DNA during serial passage of simian virus 40 at high multiplicity. *Virology* 54:384-397.
- Brockman, W. W., and D. Nathans. 1974. The isolation of simian virus 40 variants with specifically altered genomes. *Proc. Natl. Acad. Sci. U.S.A.* 71:942-946.
- Brugge, J. S., and J. S. Butel. 1975. Involvement of the simian virus 40 gene A function in the maintenance of transformation. *J. Virol.* 15:619-635.
- Chou, J. Y., and R. G. Martin. 1975. DNA infectivity and the induction of host DNA synthesis with temperature-sensitive mutants of simian virus 40. *J. Virol.* 15:145-150.
- Clark, R., D. Lane, and R. Tjian. 1981. Use of monoclonal antibodies as probes of simian virus 40 T antigen ATPase activity. *J. Biol. Chem.* 256:11854-11858.
- Clark, R., K. Peden, J. M. Pipas, D. Nathans, and R. Tjian. 1983. Biochemical activities of T-antigen proteins encoded by simian virus 40 A gene deletion mutants. *Mol. Cell. Biol.* 3:220-228.
- Clertant, P., and I. Seif. 1984. A common function for polyoma virus large-T and papilloma virus E1 proteins? *Nature (London)* 311:276-279.
- Cosman, D. J., and M. J. Trevethia. 1981. Characterization of a temperature-sensitive, DNA-positive, nontransforming mutant of simian virus 40. *Virology* 112:605-624.
- Cowan, K., P. Tegtmeyer, and D. D. Anthony. 1973. Relationship of replication and transcription of simian virus 40 DNA. *Proc. Natl. Acad. Sci. U.S.A.* 70:1927-1930.
- Friedman, T., A. Esty, P. LaPorte, and P. Deininger. 1979. The nucleotide sequence and genome organization of the polyoma early region: extensive nucleotide and amino acid homology with SV40. *Cell* 17:715-724.
- Frost, E., and J. Williams. 1978. Mapping temperature-sensitive and host-range mutations of adenovirus type 5 by marker rescue. *Virology* 91:39-50.
- Galanti, N., G. J. Jonak, K. J. Soprano, J. Floros, L. Kaczmarek, S. Weissman, V. B. Reddy, S. M. Tilghman, and R. Baserga. 1981. Characterization and biological activity of cloned simian virus 40 DNA fragments. *J. Biol. Chem.* 256:6469-6474.
- Giacherio, D., and L. P. Hager. 1979. A poly dT stimulated ATPase activity associated with SV40 large T antigen. *J. Biol. Chem.* 254:8113-8116.
- Giza, P. E., D. M. Schmit, and B. L. Murr. 1981. Region- and strand-specific mutagenesis of a recombinant plasmid. *Gene* 15:331-342.
- Gluzman, Y., and B. Ahrens. 1982. SV40 early mutants that are defective for viral DNA synthesis but competent for transformation of cultured rat and simian cells. *Virology* 123:78-92.
- Gluzman, Y., J. Davison, M. Oren, and E. Winocour. 1977. Properties of permissive monkey cells transformed by UV-irradiated simian virus 40. *J. Virol.* 22:256-266.
- Graham, F. L., and A. J. van der Eb. 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* 52:456-467.
- Hiscott, J. B., and V. Defendi. 1979. Simian virus 40 gene A regulation of cellular DNA synthesis. I. In permissive cells. *J. Virol.* 30:590-599.
- Hiscott, J. B., and V. Defendi. 1981. Simian virus 40 gene A regulation of cellular DNA synthesis. II. In nonpermissive cells. *J. Virol.* 37:802-812.
- Holmes, D. S., and M. Quigley. 1981. A rapid boiling method for the preparation of bacterial plasmids. *Anal. Biochem.* 114:193-197.
- Kalderon, D., and A. E. Smith. 1984. In vitro mutagenesis of a putative DNA binding domain of SV40 large-T. *Virology* 139:109-137.
- Keller, J. M., and J. C. Alwine. 1984. Activation of the SV40 late promoter: direct effects of T antigen in the absence of viral DNA replication. *Cell* 36:381-389.
- Khoury, G., and E. May. 1977. Regulation of early and late simian virus 40 transcription: overproduction of early viral RNA in the absence of a functional T-antigen. *J. Virol.* 23:167-176.
- Kimura, G., and R. Dulbecco. 1973. A temperature-sensitive mutant of simian virus 40 affecting transforming ability. *Virology* 52:529-534.
- Kirmura, G., and A. Itagaki. 1975. Initiation and maintenance of cell transformation by simian virus 40: a viral genetic property. *Proc. Natl. Acad. Sci. U.S.A.* 72:673-677.
- Lai, C.-J., and D. Nathans. 1975. A map of temperature-sensitive mutants of simian virus 40. *Virology* 66:70-81.
- Lane, D. P., and L. V. Crawford. 1979. T antigen is bound to a host protein in SV40-transformed cells. *Nature (London)* 278:261-263.
- Lederberg, E. M., and S. N. Cohen. 1974. Transformation of *Salmonella typhimurium* by plasmid deoxyribonucleic acid. *J. Bacteriol.* 119:1072-1074.
- Li, J. J., and T. J. Kelly, Jr. 1984. Simian virus 40 DNA replication in vitro. *Proc. Natl. Acad. Sci. U.S.A.* 81:6973-6977.
- Linzer, D. I. H., and A. J. Levine. 1979. Characterization of a 54K dalton cellular SV40 tumor antigen present in SV40-transformed cells and uninfected embryonal carcinoma cells. *Cell* 17:43-52.
- Maniatis, T., A. Jeffrey, and D. G. Kleid. 1975. Nucleotide sequence of the rightward operator of phage λ . *Proc. Natl. Acad. Sci. U.S.A.* 72:1184-1188.
- Manos, M. M., and Y. Gluzman. 1984. Simian virus 40 large T-antigen point mutants that are defective in viral DNA replication but competent in oncogenic transformation. *Mol. Cell. Biol.* 4:1125-1133.
- Manos, M. M., and Y. Gluzman. 1985. Genetic and biochemical analysis of transformation-competent, replication-defective simian virus 40 large T antigen mutants. *J. Virol.* 53:120-127.
- Margolske, R. F., and D. Nathans. 1984. Simian virus 40 mutant T antigens with relaxed specificity for the nucleotide sequence at the viral DNA origin of replication. *J. Virol.* 49:386-393.
- Martin, R. G., and J. Y. Chou. 1975. Simian virus 40 functions required for the establishment and maintenance of malignant transformation. *J. Virol.* 15:599-612.
- Maxam, A. M., and W. Gilbert. 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. *Methods Enzymol.* 65:499-560.
- McCutchan, J. H., and J. S. Pagano. 1968. Enhancement of the infectivity of simian virus 40 deoxyribonucleic acid with diethylaminoethyl-dextran. *J. Natl. Cancer Inst.* 41:351-357.
- McKay, R., and D. DiMaio. 1981. Binding of an SV40 T antigen-related protein to the DNA of SV40 regulatory mutants. *Nature (London)* 289:810-813.
- McKay, R. D. G. 1981. Binding of a simian virus 40 T antigen-

- related protein to DNA. *J. Mol. Biol.* 145:471-488.
43. Meselson, M., and R. Yuan. 1968. DNA restriction enzyme from *E. coli*. *Nature (London)* 217:1110-1114.
 44. Osborn, M., and K. Weber. 1975. Simian virus 40 gene A function and maintenance of transformation. *J. Virol.* 15:636-644.
 45. Parker, B. A., and G. R. Stark. 1979. Regulation of simian virus 40 transcription: sensitive analysis of the RNA species present early in infections by virus or viral DNA. *J. Virol.* 31:360-369.
 46. Peden, K., P. Mounts, and G. S. Hayward. 1982. Homology between mammalian DNA sequences and human herpesvirus genomes detected by a hybridization procedure with high-complexity probe. *Cell* 31:71-80.
 47. Peden, K. W. C., and D. Nathans. 1982. Local mutagenesis within deletion loops of DNA heteroduplexes. *Proc. Natl. Acad. Sci. U.S.A.* 79:7214-7217.
 48. Peden, K. W. C., J. M. Pipas, S. Pearson-White, and D. Nathans. 1980. Isolation of mutants of an animal virus in bacteria. *Science* 209:1392-1396.
 49. Pipas, J. M., K. W. C. Peden, and D. Nathans. 1983. Mutational analysis of simian virus 40 T antigen: isolation and characterization of mutants with deletions in the T-antigen gene. *Mol. Cell. Biol.* 3:203-213.
 50. Pope, J. H., and W. P. Rowe. 1964. Detection of specific antigen in SV40-transformed cells by immunofluorescence. *J. Exp. Med.* 120:121-128.
 51. Prives, C., L. Covey, A. Schellier, and Y. Gluzman. 1983. DNA-binding properties of simian virus 40 T-antigen mutants defective in viral DNA replication. *Mol. Cell. Biol.* 3:1958-1966.
 52. Reed, S. I., J. Ferguson, R. W. Davis, and G. R. Stark. 1975. T antigen binds to SV40 at the origin of DNA replication. *Proc. Natl. Acad. Sci. U.S.A.* 72:1605-1609.
 53. Reed, S. I., G. R. Stark, and J. C. Alwine. 1976. Autoregulation of simian virus 40 gene A by T antigen. *Proc. Natl. Acad. Sci. U.S.A.* 73:3083-3087.
 54. Rigby, P. W. J., M. Dieckmann, C. Rhodes, and P. Berg. 1977. Labelling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. *J. Mol. Biol.* 113:237-251.
 55. Rosenthal, L. J., and M. Brown. 1977. The control of SV40 transcription during lytic infection: late RNA synthesis in the presence of inhibitors of DNA replication. *Nucleic Acids Res.* 4:551-565.
 56. Sanger, F., and A. R. Coulson. 1978. The use of thin acrylamide gels for DNA sequencing. *FEBS Lett.* 87:107-110.
 57. Scott, W. A., W. W. Brockman, and D. Nathans. 1976. Biological activities of deletion mutants of simian virus 40. *Virology* 75:319-334.
 58. Shalloway, D., T. Kleinberger, and D. M. Livingston. 1980. Mapping of SV40 DNA replication origin region binding sites for the SV40 T antigen by protection against exonuclease III digestion. *Cell* 20:411-422.
 59. Shiroki, K., and H. Shimojo. 1971. Transformation of green monkey kidney cells by SV40 genome; the establishment of transformed cell lines and the replication of human adenovirus and SV40 in transformed cells. *Virology* 45:163-171.
 60. Shortle, D., and D. Nathans. 1978. Local mutagenesis: a method for generating viral mutants with base substitutions in preselected regions of the viral genome. *Proc. Natl. Acad. Sci. U.S.A.* 75:2170-2174.
 61. Shortle, D., and D. Nathans. 1979. Regulatory mutants of simian virus 40: constructed mutants with base substitutions of the origin of viral DNA replication. *J. Mol. Biol.* 131:801-817.
 62. Shortle, D. R., R. F. Margolskee, and D. Nathans. 1979. Mutational analysis of the simian virus 40 replicon: pseudorevertants of mutants with a defective replication origin. *Proc. Natl. Acad. Sci. U.S.A.* 76:6128-6131.
 63. Smith, H. O. 1980. Recovery of DNA from gels. *Methods Enzymol.* 65:371-380.
 64. Soprano, K. J., V. G. Dev, C. M. Croce, and R. Baserga. 1979. Reactivation of silent rRNA genes by simian virus 40 in human-mouse hybrid cells. *Proc. Natl. Acad. Sci. U.S.A.* 76:3885-3889.
 65. Soprano, K. J., N. Galanti, G. J. Jonak, S. McKercher, J. M. Pipas, K. W. C. Peden, and R. Baserga. 1983. Mutational analysis of simian virus 40 T antigen: stimulation of cellular DNA synthesis and activation of rRNA genes by mutants with deletions in the T-antigen gene. *Mol. Cell. Biol.* 3:214-219.
 66. Soprano, K. J., M. Rossini, C. Croce, and R. Baserga. 1980. The role of large T antigen in SV40-induced reactivation of silent rRNA genes in human-mouse hybrid cells. *Virology* 102:317-326.
 67. Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* 98:503-517.
 68. Steinberg, B., R. Pollack, W. Topp, and M. Botchan. 1978. Isolation and characterization of T antigen-negative revertants from a line of transformed rat cells containing one copy of the SV40 genome. *Cell* 13:19-32.
 69. Stringer, J. R. 1982. Mutant of simian virus 40 large T-antigen that is defective for viral DNA synthesis, but competent for transformation of cultured rat cells. *J. Virol.* 42:854-864.
 70. Suarez, H. G., M. F. Bourali, R. Wicker, S. Estrada, and C. Cassingena. 1972. Properties of an SV40-transformed African green monkey kidney (BSC-1) cell line. *Int. J. Cancer* 9:324-333.
 71. Tegtmeier, P. 1972. Simian virus 40 deoxyribonucleic acid synthesis: the viral replicon. *J. Virol.* 10:591-598.
 72. Tegtmeier, P. 1975. Function of simian virus 40 gene A in transforming infection. *J. Virol.* 15:613-618.
 73. Tegtmeier, P., M. Schwartz, J. K. Collins, and K. Rundell. 1975. Regulation of tumor antigen synthesis by simian virus 40 gene A. *J. Virol.* 16:168-178.
 74. Tjian, R. 1978. The binding site on SV40 DNA for a T antigen-related protein. *Cell* 13:165-179.
 75. Tjian, R., G. Fey, and A. Graessmann. 1978. Biological activity of purified simian virus 40 T antigen proteins. *Proc. Natl. Acad. Sci. U.S.A.* 75:1279-1283.
 76. Tjian, R., and A. Robbins. 1979. Enzymatic activities associated with a purified simian virus 40 T antigen-related protein. *Proc. Natl. Acad. Sci. U.S.A.* 76:610-614.
 77. Todaro, G. J., and H. Green. 1976. An assay for cellular transformation by SV40. *Virology* 23:117-119.
 78. Toozé, J. 1981. DNA tumor viruses: molecular biology of tumor viruses, 2nd ed., rev., p. 799-841. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
 79. Topp, W. C. 1981. Normal rat cell lines deficient in nuclear thymidine kinase. *Virology* 113:408-411.
 80. Van Roy, F., L. Fransen, and W. Fiers. 1983. Improved localization of phosphorylation sites in simian virus 40 large T antigen. *J. Virol.* 45:315-331.